2683

FURTHER STUDIES ON THE BEHAVIOR OF SOME NON-NITROGENOUS COMPOUNDS WITH NINHYDRIN DURING AUTOMATIC ION EXCHANGE CHROMATOGRAPHY

SCHILLING *et al.*[1] and ZACHARIUS AND TALLEY[2] reported the reaction of non-nitrogenous keto acids and lactones related to carbohydrates, with ninhydrin following ion exchange chromatography. Elution volumes of the compounds were given. Also on chromatographing acid hydrolysates of non-protein and crude protein preparations of potato tuber an unknown peak was observed from which levulinic acid was isolated and identified[2]. A structure for the ninhydrin–levulinic acid reaction was later proposed[3] and the list of non-nitrogenous compounds which react with ninhydrin was expanded[3,4].

As a further extension of this work, two peaks with very low elution volumes were noted in ion exchange chromatography of a non-nitrogenous fraction of the red tart cherry (*Prunus cerasus*). This paper reports the isolation and identification of glucose and fructose as the compounds responsible for these peaks. Also reported are the reaction with ninhydrin and the elution volumes of a number of other carbohydrates and related compounds, most of which have not hitherto been examined.

EXPERIMENTAL

Non-protein nitrogen extracts of the red tart cherry were prepared by 80% aqueous ethanol extraction, concentrated *in vacuo* at temperatures not exceeding 40° and made to volume in water. Aliquots were applied to the analytical column following 1.0 ml of 0.2 *N* pH 2.2 citrate buffer.

Analytical separations of the non-protein nitrogen fraction on an Amberlite IR-120 resin column revealed a small unidentified peak at 55 ml and indication of another at 50 ml (Fig. 1). The reaction product(s) produced a much greater absorption at 440 mμ than at 570 mμ. A preparative column was used for the isolation of the unreacted material[2,6] which was located by assaying small aliquots of the collected fractions with ninhydrin[7]. The fractions containing the unknown were desalted[8] and concentrated. Rechromatography on the analytical column revealed a major peak at 55–60 ml with maximum absorption at 440 mμ; a number of considerably smaller peaks were also visible.

The isolated material afforded a positive osazone reaction. When spotted on filter paper, positive sugar reactions with both *p*-anisidine hydrochloride and benzidine reagent were observed[9]. Unidirectional paper chromatography with *n*-butanol–acetic acid–water (9:1:2.5) revealed three spots with both *p*-anisidine and benzidine reagents, two of which corresponded with the R_F of glucose and fructose. The presence

of a ketose was further corroborated with naphthoresorcinol reagent[10]. The third spot, which was not identified, did not correspond to a common hexose and failed to give a pentose reaction with phloroglucinol. Small amounts of the two sugars were separated

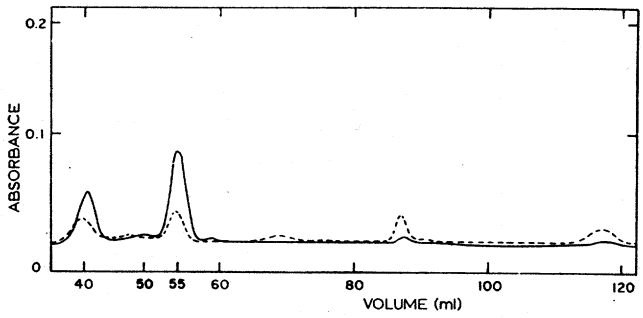

Fig. 1. Unidentified 440 mμ ninhydrin peaks from the non-protein nitrogen fraction of red tart cherries on the amino acid analyzer. Amberlite IR-120 column, 30°, pH 3.25, 0.2 *N* Na citrate. (———) 440 mμ; (– – –) 570 mμ.

and isolated from the column fraction by chromatography on acid-washed filter paper sheets with the acid–butanol solvent. A phenylosazone derivative, prepared from each, gave similar melting points of 204–205° with no depression on mixing. Although the phenylosazone of D-mannose has a melting point identical with that of D-glucose and D-fructose, the absence of D-mannose in the column fraction was shown by paper strip chromatography employing γ-collidine saturated with water.

Chromatography of a standard mixture of D-glucose and D-fructose on the amino acid analyzer under conditions as previously described, produced two closely adjoining peaks. Both had a maximum considerably greater at 440 mμ than at 570 mμ but the glucose was only 1/18 as sensitive to ninhydrin as fructose. Chromatography of the isolated material with fructose and glucose standards produced a peak pattern indistinguishable from that of the isolated mixture alone.

A number of other sugars and related compounds were chromatographed on the analyzer as described above. All compounds were from commercial sources except xylulose which was prepared by epimerization of D-xylose. With the exception of acrolein and xylulose all compounds were determined to be pure by means of paper and ion exchange chromatography and gas chromatography of their trimethylsilyl derivatives. The ability of these compounds to produce colored products, the 440:570 mμ absorption ratio of the products, elution volumes and approximate color factors were ascertained. The colored reaction products of the ninhydrin reagent with levulinic acid, fructose, glyceraldehyde, dihydroxyacetone and ascorbic acid were examined on the Cary ultraviolet spectrophotometer before and after oxygen was bubbled through the solution for 30 sec. The compounds were reacted in test tubes with MOORE AND STEIN ninhydrin reagent[7].

RESULTS AND DISCUSSION

The finding of fructose and glucose in extracts of the cherry is not surprising but this would appear to be the first time that the compounds have been discerned as

peaks during the amino acid analysis of a natural material and their identity established. It has been reported[3] that fructose can produce a peak with ninhydrin on the amino acid analyzer in studies with a standard solution of the sugar, although glucose was considered non-reacting[3,4] and was not expected to be revealed.

The low sensitivity of fructose (compared with amino acids) would not cause a significant error with plant extracts having a high amino acid-reducing sugar ratio (*e.g.* potato tuber) but in extracts having a low ratio (*e.g.* cherry fruit), an error in interpretation might result.

The behavior of these and other non-nitrogenous compounds which produce peaks with ninhydrin on the amino acid analyzer are compiled in Table I and their positions are schematically shown on a composite elution pattern (Fig. 2).

TABLE I

NINHYDRIN PEAKS WITH SOME NON-NITROGENOUS COMPOUNDS ON AN AMINO ACID ANALYZER

Code No. Fig. 2	*Compound*	*Elution volume*	*440:570 mμ absorption ratio*	*Color factor*
1	Turanose	45	2.0	0.00081
2	Lactose	45	2.0	0.00063
3	Glucose	51–52	3.3	0.00072
4	Galactose	54	3.3	0.00093
5	Mannose	54	3.3	0.0010
6	Sorbose	54–55	5.69	0.026
7	Rhamnose	57–58	3.3	0.00099
8	Fructose	57–58	5.23	0.0132
9	Glyceraldehyde (dimer)[a]	56–58	1.29→2.55	0.178→0.016
10	Glyceraldehyde (monomer)[a]	62–64	2.59←1.23	0.029←0.139
11	Ascorbic acid	65–66	2.57–3.94	0.384–0.440
12	Ribose	69	4.71	0.0084
13	Methylglyoxal	68–69	2.2	—
14	Hydroxymethylfurfural	76–77	25.	—
15	Dihydroxyacetone[a]	78–79	1.20→2.27	0.087←0.193
16	Levulinic acid	79–80	1.34	0.250
17	Hydroxyproline[b]	115–116	5.95	1.58
18	Kojic acid	137–139	4.28	0.63
19	Xylose	58–59	4.0	0.0027
20	Arabinose	62–63	5.1	0.0020
21	Lyxose	58–59	2.72	0.014
22[1,2]	Xylulose[c]	1. 57–59	1. 2.19–5.09	—
		2. 64–67	2. 5.0 –10.0	—
23	Acetaldehyde	70.5	4.5	0.0016
24	Crotonaldehyde	113	18.	0.028
25A	Glycoaldehyde (dimer)	62.5–64.5	1.56	0.012
25B	Glycoaldehyde (monomer)	64–66	1.70	0.026
26[1,2,3]	Acrolein[c]	1. 64.5	1. 440 only	—
		2. 79.5	2. 3.4	—
		3. 104	3. 440 only	—

[a] For an explanation of arrows, see text.
[b] Nitrogenous compound for reference.
[c] More than one peak was observed. See text.

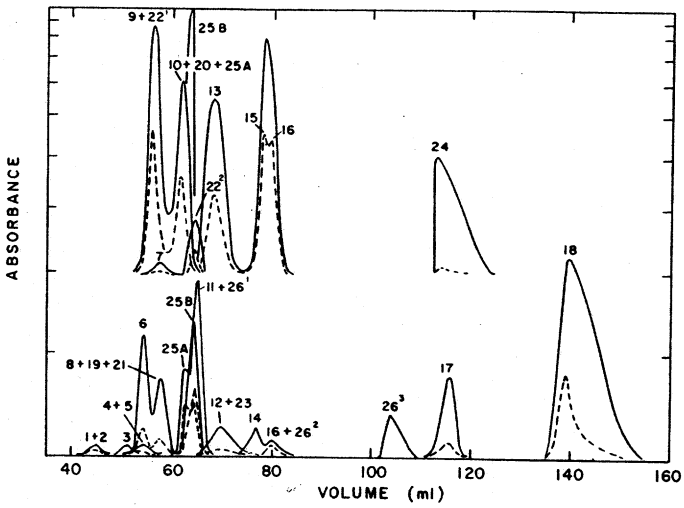

Fig. 2. Elution volumes of some non-nitrogenous compounds yielding a peak on the amino acid analyzer. 150 × 0.9 cm Amberlite IR-120 column, 30°, 0.2*N* Na citrate. (———) 440 mμ; (– – –) 570 mμ.

None of the compounds listed in Table I and Fig. 2 contains nitrogen except for hydroxyproline which was included for reference because of its elution volume and 440 mμ color absorption of its ninhydrin reaction product. All of the non-nitrogenous compounds described produce peaks with a 440:570 mμ absorption ratio greater than 1.0. The color factors, however, showed a range of 1000-fold from lactose to kojic acid.

SCHILLING *et al.*[3] had reported a list of nitrogen-free compounds which yielded a ninhydrin-positive reaction on the amino acid analyzer. However, the compounds chosen were first subjected to a pretest outside of the closed system analyzer at which time air was bubbled through the reactant products. This was done to eliminate the possibility of a simple ninhydrin reduction with an accompanying false color production. If a "positive" reaction was noted, the compound was then examined for its behavior on the amino acid analyzer. The anaerobic conditions of the latter, however, would not exclude the "false color production" which might result with an unknown constituent. By these criteria, SCHILLING *et al.* consider that glucose and 5-hydroxymethylfurfural are compounds which do not give a positive ninhydrin reaction. Yet, when run on the amino acid analyzer at high enough concentration, the present study reveals peaks which can be attributed to them (Table I and Fig. 2). The term positive ninhydrin reaction with non-nitrogenous compounds is only relative and meaningful in the light of the conditions employed. One notes that HU *et al.*[4], although concerned with 570 mμ absorption products, report no color was produced when fructose, sorbose, glucose, ribose and xylose were reacted with ninhydrin. The present investigation demonstrates peaks on the analyzer for all five of the aforementioned compounds, and SCHILLING *et al.* included fructose in their ninhydrin-positive list.

In this study the non-reducing sugars, sucrose, melezitose and raffinose at a level as high as 72 mg per analysis failed to produce a ninhydrin peak on the analyzer. Chlorogenic acid (19 mg), caffeic acid (4 mg) and oxalic acid (6 mg) also proved negative.

Examination with the Cary spectrophotometer of the reddish-brown ninhydrin reaction products of levulinic acid, ascorbic acid, fructose, dihydroxyacetone and glyceraldehyde before and after treatment with molecular oxygen indicated that of the five compounds studied, only the levulinic acid product was stable (Table II). It showed stability on standing, with 97–98 % of the color remaining after 40 min and no

TABLE II

ABSORPTION CHARACTERISTICS OF NINHYDRIN-COLORED PRODUCT BEFORE AND AFTER OXYGENATION

Compound	*Absorption maximum on Cary (mμ)*	*440:570 mμ absorption ratio before O_2*	*440:570 mμ absorption ratio after O_2*	*Alteration of absorption curve*
Levulinic acid	525	1.28	1.31	Shape unchanged; slightly lowered absorption
Fructose	475	2.20	8.50	Shape changed with max. disappearing
Ascorbic acid	550	1.29	1.45	Initial curve resembles elevated ninhydrin blank; lowered but shape unchanged by O_2
Dihydroxyacetone	480	2.06	2.53	Shape changed with O_2, max. disappearing; resembled elevated ninhydrin blank
Glyceraldehyde (dimer and monomer)	475–480	1.73	1.97	Shape changed with O_2, max. disappearing; resembled elevated ninhydrin blank

change in the 440:570 mμ ratio. Moreover, oxygenation only slightly affected the ratio along with a small depression in absorbancy. On the other hand, the reaction products of the other compounds faded on standing and showed a large alteration in their 440:570 mμ ratio on oxygenation. The results are summarized in Table II. LAGERCRANTZ AND YHLAND[11] have attributed a connection between the reddish-brown colored products with free ninhydrin radicals obtained from glyoxal or dithionite reacted with ninhydrin under slightly alkaline conditions but it was not clear whether the colored substances were identical with the radicals. However, the color faded and radicals decreased with shaking in air, indicating both are destroyed by oxygen. While LAGERCRANTZ AND YHLAND report no free radicals could be detected below pH 6.5, they carried out the reaction in a flat solution cell presumably exposed to air. The ninhydrin reaction described in the present study occurs at pH 5.5 under near anaerobic conditions on the amino acid analyzer; conceivably, the latter may alter the pH requirements for free radical formation. The findings of HU *et al.*[4] would tend to support this idea.

Alteration in the 440:570 mμ absorption ratio of the four compounds studied here might be explained from the fact that either or both of these spectrum points are

located on a steep slope of their curves. The shape of the curve was found to be subject to the concentration of the compound and could be altered by oxygen.

The color factors were determined on the closed system analyzer, largely free of air, and were found to be relatively constant for fructose in the 8–40 mg range studied. For ascorbic acid it proved to be somewhat erratic (± 9 % deviation) in the 0.5–5.0 mg range and this could not be ascribed to concentration effects. Dihydroxyacetone produced both erratic color values and absorption ratios. Neither appeared to be related to concentration but an inverse relation was noted between the direction of the two. This is indicated in Table I by the direction of the arrows.

DL-Glyceraldehyde produced two peaks on the amino acid analyzer which presumably represent the monomer and dimer. The dimers of glycoaldehyde and glyceraldehyde have been formulated to be products of an extramolecular acetal formation analogous to the formation of pyranose and furanose rings by the higher sugars[12]. In aqueous solution the glyceraldehyde dimer is gradually converted to the monomeric form[13]. When freshly dissolved DL-glyceraldehyde was chromatographed on the analyzer, two peaks were observed, separated by a 6 ml elution volume (Table I, Fig. 2), and the faster peak (dimer) is predominant. However, when the glyceraldehyde solution is allowed to stand for several hours before separation on the column, the slower moving component (monomer) is found to have increased at the expense of the faster one. On further standing (two days) the monomer peak predominates on chromatography. Although SCHILLING *et al.*[3] have indicated a single peak for glyceraldehyde, at no time did our aqueous solutions prepared from two different commercial sources of the compound demonstrate a single peak. Gas chromatography of the trimethylsilyl derivative which is prepared in a non-aqueous medium produced a single peak. It would seem that the monomer–dimer forms exist in equilibrium in aqueous solution or pH 3.25 citrate buffer. Unfortunately, the color factors do not indicate the ninhydrin reagent to be especially suitable for following the conversion of dimer to monomer in a quantitative manner, although the column conditions are suitable for resolving them. An inverse relation, as in the case of dihydroxyacetone, was observed between the absorption ratios and color factors of the ninhydrin products of both monomer and dimer (see arrows in Table I). At the lower concentrations of glyceraldehyde examined, these values were somewhat erratic and also in many cases failed to yield a reproducible monomer–dimer ratio.

Glycoaldehyde yielded two peaks, incompletely resolved, with a 1.5 ml elution volume difference. By analogy with the behavior of glyceraldehyde, the dimer is assumed to be the faster moving (and smaller) of the two peaks. Permitting an aqueous solution to stand at room temperature up to six days does not measurably change the distribution of the monomer–dimer peaks. In the case of glycoaldehyde as compared with glyceraldehyde, the fresh aqueous solution contains a preponderance (2.5 times) of monomer if equal color factors are assumed. As in the case of glyceraldehyde the trimethylsilyl derivative produced a single peak on gas chromatography. Glycoaldehyde and glyceraldehyde, although having closely related elution volumes, are resolved on the analyzer.

The peaks from fructose, lyxose and xylose are perfectly superimposed on each other. It is of interest that the closely related aldopentose sugars do not yield the same color factor with ninhydrin. Xylose and arabinose have an analogous but lower value than ribose and lyxose. The latter two sugars contain a pair of *cis* hydroxyl groups

adjacent to the carbonyl, perhaps offering greater opportunity for free radical formation in the ninhydrin reaction. Moreover, it is well known that similar differences in sugar structure (*cis vs. trans* hydroxyl configuration) affect reactivity with lead tetraacetate and periodic acid.

The three peaks derived from a commercial preparation of acrolein reflect the highly reactive nature of the compound. They have been included on Fig. 2 because the peaks may be encountered where the dehydration of glycerol occurs.

All of the reaction products observed in this and the earlier study[2] between non-nitrogenous compounds and ninhydrin on the amino acid analyzer have had greater absorption at 440 mμ (*vs.* 570 mμ). Recently, Hu *et al.*[4] have reported that erythrulose, xylulose, and ribulose reacted with ninhydrin reagent[7] to produce a peak having an absorption maximum at 570 mμ with a sensitivity approximately 1/50 that of leucine. This unorthodox behavior of nitrogen-free compounds was of much interest to this investigation. Hence, xylulose was prepared by epimerization of D-xylose[14] and the preparation freed of much residual xylose by crystallization. The product produced six peaks on the analyzer, all with the greater absorption at 440 mμ. Two of the six peaks were of major size (22^1 and 22^2 in Table I), the former being very sharp. Only these two peaks are presented in Table I and Fig. 2. Following purification on paper sheets with two separate solvent systems, the product produced no appreciable change on the analyzer. The reaction product was separated on acid-washed Whatman 3MM paper with isopropanol–water (160:40) and the position of the ketopentose clearly located with naphthoresorcinol or phloroglucinol reagents. The carefully demarcated xylulose area was removed, eluted, filtered through a thin layer of decolorizing charcoal, and concentrated to a clear, almost colorless, syrup. Paper chromatographic purification was repeated using ethyl acetate–pyridine–water (120:50:40) on the once purified material.

Paper chromatography of the twice purified material with isopropanol–water (160:40) revealed three spots with benzidine reagent, one of which also gave a positive ketose reaction with phloroglucinol. One of the contaminants had an R_F identical with both xylose and lyxose. Although peak 22^1 does have an elution volume in agreement with both of these sugars, its very sharp separation on the column is not analogous with the behavior of xylose or lyxose. Studied on the analyzer after each purification, the original six peaks were observed in approximately the same ratio. Both paper and ion exchange chromatography have eliminated glyceraldehyde, dihydroxyacetone and glycoaldehyde as components of the mixture. Gas chromatography yielded one major and minor peak along with some trace peaks, and completely eliminated ribose, lyxose and, notably, xylose. While the minor peak cochromatographed with a similar elution time as arabinose, its identity with this pentose was eliminated on paper chromatography. It is suggested that the components may exist in equilibrium under the conditions employed in order to explain the unchanged analytical results following purification. Hu *et al.*[4] have reported only a single peak for xylulose with an absorption maximum at 570 mμ. It appears, however, that these investigators have carried out their analytical separations using a manual procedure with the accompanying exposure to air as compared with the closed system amino acid analyzer used in this study. In fact, they have described no color production when D-fructose, L-sorbose, etc. were treated with ninhydrin under the same conditions. In our study, these same sugars yielded a peak on the amino acid analyzer with a maximum absorption at 440

mμ but when the ninhydrin reaction was carried out in a test tube on fructose, etc. the color faded to an elevated ninhydrin blank. Moreover, the manual method provides an uneven blank (base line) in which small peaks might not be discerned.

On the analyzer, the 440:570 mμ ratio for at least peak 22[1] (xylulose) varies considerably with the level of sugar reactant. When the absorbance of peak 22[1] was 0.28, the absorption ratio was 4.4, at 1.0, 2.2, and at 1.5, 1.6. Conceivably, at higher levels of xylulose, the ratio might become less than 1.0. Nevertheless, our own study of the behavior of the xylulose preparation with ninhydrin in the test tube and examination on the Cary of the spectral properties of the reaction product showed a maximum absorption at 470 mμ rather than 570 mμ. HU *et al.*[4] have not described the level of xylulose employed.

ACKNOWLEDGEMENTS

We wish to thank Mr. JESSE ARD and Mr. CARL LEANDER, Jr. for their technical assistance with the Cary ultraviolet scans and Dr. I. SCHMELTZ for his assistance with the gas chromatograph. We are also grateful to Dr. PAUL B. HAMILTON for his helpful criticism of this manuscript.

SUMMARY

Automatic amino acid analysis of cherry extract revealed an unidentified ninhydrin peak. Fractions representing this peak were subsequently isolated and the contents characterized as a mixture containing glucose and fructose. Further investigations are presented on the behavior of a number of carbohydrates and related non-nitrogenous compounds on the amino acid analyzer. Certain differences in these findings and those in the literature are pointed out and discussed. Monomer–dimer relationship in solutions of glyceraldehyde and glycoaldehyde are demonstrated. The nature of the absorption curve resulting from the reaction of ninhydrin with some of the nitrogen-free compounds was examined. Possible anomalies between results obtained on the amino acid analyzer and the manual procedure are discussed. Results from this and previous studies reiterate earlier warnings on the hazards of interpretation of all ninhydrin peaks as products of nitrogen compounds.

REFERENCES

1 E. D. SCHILLING, P. I. BURCHILL, J. R. COFFMAN AND R. A. CLAYTON, *Biochem. Biophys. Res. Commun.*, 5 (1961) 217.
2 R. M. ZACHARIUS AND E. A. TALLEY, *J. Chromatog.*, 7 (1962) 51.
3 E. D. SCHILLING, P. I. BURCHILL AND R. A. CLAYTON, *Anal. Biochem.*, 5 (1963) 1.
4 C. L. HU, V. V. RENDIG AND E. A. MCCOMB, *J. Chromatog.*, 19 (1965) 622.
5 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
6 E. A. TALLEY AND W. L. PORTER, *J. Chromatog.*, 3 (1960) 434.
7 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 907.
8 E. SCHRAM, J. P. DUSTIN, S. MOORE AND E. J. BIGWOOD, *Anal. Chim. Acta*, 9 (1953) 149.
9 R. H. HORROCKS, *Nature*, 164 (1949) 444.
10 G. HARRIS AND I. C. MACWILLIAM, *Chem. Ind. (London)*, (1954) 249.
11 C. LAGERCRANTZ AND M. YHLAND, *Acta Chem. Scand.*, 17 (1963) 277.
12 W. PIGMAN, *The Carbohydrates*, Academic Press, New York, 1957, pp. 69–70.
13 A. WOHL, *Ber.*, 31 (1898) 2394.
14 O. TOUSTER, in R. L. WHISTLER AND M. L. WOLFROM (Editors), *Methods in Carbohydrate Chemistry*, Vol. 1, Academic Press, New York, 1962, pp. 98–101.